

# Biochemical characterization of indigenous green microalgae and FAMES profiling of lipids for sustainable biodiesel production

Olufunke Oyebamiji<sup>1\*</sup>, Matthew Ilori<sup>2</sup>, Olukayode Amund<sup>2</sup>, Solomon Olaleru<sup>3</sup> and Wiebke Boeing<sup>4</sup>

<sup>1</sup>Department of Biological Sciences, Yaba College of Technology, Lagos, Nigeria

<sup>2</sup>Department of Microbiology, University of Lagos, Akoka, Yaba, Nigeria

<sup>3</sup>Department of Physical Sciences, Yaba College of Technology, Lagos, Nigeria

<sup>4</sup>Department of Fish, Wildlife, and Conservation Ecology, New Mexico State University, Las Cruces, NM 88003, USA

**Abstract.** There is a growing need for sustainable energy sources, which has led to increased interest in microalgae as a feedstock for the production of biodiesel. This study investigated indigenous green microalgae for biodiesel generation through comprehensive biochemical and analytical evaluations of their biomass. Green microalgae species were cultured in BG-11 medium under controlled photobioreactor conditions, harvested, and analyzed for protein, carbohydrate, lipid, and Carbon-Hydrogen-Nitrogen-Sulphur (CHNS) composition. Nile red staining and Bligh and Dyer extraction revealed lipid content ranging from 11.74 - 32.17 % and *in-situ* transesterification and GC-MS profiling quantified fatty acid methyl esters (FAMES). *Chlorella* sp. MOW 3 showed the highest yield (4.48 % of biomass). Protein and carbohydrate content varied between 17.76 – 47.43 % and 13.59 – 46.56 %, respectively. The CHNS analysis showed the highest carbon content in *Graesiella emersonii* MOW 9 (55.55 %). Only lipid content exhibited a statistically significant correlation with the C/N ratio, and the CHNS analysis directly validates lipid metabolic regulation in these isolates. GC-MS analysis revealed saturated, monounsaturated, and polyunsaturated fatty acids, with palmitic acid, oleic acid, and linoleic acid being the most abundant. These findings underscore the viability of indigenous microalgae strains as promising candidates for renewable biodiesel production.

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\* Corresponding author: [olufunke.oyebamiji@yabatech.edu.ng](mailto:olufunke.oyebamiji@yabatech.edu.ng)

## 1 Introduction

The global demand for sustainable and renewable energy sources has increased due to the environmental impacts of fossil fuel consumption. The consequences include greenhouse gas emissions, climate change, and resource depletion. Biodiesel, a biodegradable and non-toxic alternative to petroleum diesel, has emerged as a promising substitute; however, conventional feedstocks such as soybean, palm, and rapeseed compete with food crops and arable land, raising concerns about food security and sustainability [1, 2].

Microalgae have garnered considerable attention as a viable and sustainable source of biodiesel due to their rapid growth rates, high lipid content, and ability to thrive on non-arable land and wastewater. Unlike terrestrial crops, microalgae can produce 10 to 100 times more oil per acre and can be cultivated year-round under controlled conditions [3, 4]. Moreover, certain strains of microalgae have demonstrated significant levels of fatty acids—particularly saturated and monounsaturated fatty acids—that are desirable for high-quality biodiesel production [5, 6].

Despite their potential, the commercial viability of microalgal biodiesel is hindered by high production costs, low biomass yields in some species, and challenges in downstream processing, including harvesting, lipid extraction, and transesterification. To address these limitations, recent studies have focused on optimizing culture conditions, developing cost-effective photobioreactors, and enhancing lipid productivity through genetic and metabolic engineering [7, 8]. Additionally, advancements in analytical techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) and *in-situ* transesterification methods have improved the precision of fatty acid methyl esters (FAMES) profiling, facilitating better assessment of biodiesel quality and yield [9].

This study hypothesizes that these indigenous green microalgae isolates cultivated in a photobioreactor possess lipids, favourable fatty acid profiles, and high carbon sequestration potential, making them an efficient and sustainable feedstock for biodiesel production. The objective is to evaluate the biodiesel production potential of selected indigenous green microalgae isolates through biochemical composition analysis, lipid extraction, and fatty acid methyl ester (FAME) profiling.

## 2 Materials and Methods

### 2.1 Mini Photobioreactor Construction

Erlenmeyer flasks were built into a mini photobioreactor by sealing with a Teflon cork with two bored holes. Two glass tubes of different lengths were cut and flamed to fit into the two holes (the longer one was used to bubble air /CO<sub>2</sub> into the culture, and the shorter one was for the O<sub>2</sub> outlet). The two glass tubes were fitted with 0.2 µm polytetrafluorethylene (PTFE) membrane filters (Sartorius) for air purification.

### 2.2 Culturing for biomass and harvesting

A total of 12 strains of freshwater green microalgae were used in this project. Their isolation, purification and molecular identification were carried out according to [10]. These were cultured using BG-11 as batch cultures in a mini photobioreactor. The setup was incubated in a Microprocessor-controlled low-temperature illuminated incubator – model 818 (ThermoFisher Scientific, USA). Culture conditions included an initial pH of 7, temperature of 26 °C, constant illumination with cool-white fluorescent bulbs at a fluency rate of 50 µmol m<sup>-2</sup>s<sup>-1</sup>. Agitation was achieved with continuous shaking on an Aros 160 adjustable reciprocating orbital shaker (Thermolyne, USA) at 110 rpm (revolutions per minute) and bubbling of atmospheric air (filtered) with PETCO air-pump (AC-9904) [11]. Biomass produced was harvested at day 14 by centrifuging at 10,000 xg for 20 minutes. The supernatant was decanted, and the pellets were flash-frozen in liquid nitrogen and lyophilized for 3 days at 50 °C using a freeze-dry system (Labconco Corporations, USA).

### 2.3 Protein Analysis

Protein was quantified using the Bio-Rad Bradford assay with bovine serum albumin (BSA) as the standard. Lyophilized microalgal biomass (5 mg) was precipitated with 24% (w/v) trichloroacetic acid (TCA), heated at 95 °C for 15 min, centrifuged, and the pellet solubilized in 0.1 M NaOH at 55 °C for 3 h. Absorbance was measured at 595 nm. A seven-point BSA calibration curve (0.05–1.00 mg/mL) was prepared in triplicate and showed linearity with R<sup>2</sup> ≥ 0.995. Limits of detection (LOD) and quantification (LOQ) were calculated using 3.3σ/slope and 10σ/slope, respectively. Spike recovery ranged between 92–104%, confirming good method accuracy [12, 13].

### 2.4 Carbohydrate Analysis

Total carbohydrate content was quantified using the MBTH (3-methyl-2-benzothiazolinone hydrazone) colorimetric assay following acid hydrolysis. Dried microalgal biomass (50 mg) was hydrolyzed with concentrated sulphuric acid, diluted with ultra-pure water, autoclaved, neutralized using calcium carbonate, and filtered. The filtrate was reacted with NaOH and MBTH, heated at 90 °C for 15 min, and subsequently treated with ferric reagent to develop colour. Absorbance was measured at 425 nm. Glucose standards (2.5–100 µg/mL) were prepared in triplicate for calibration, yielding excellent linearity (R<sup>2</sup> ≥ 0.993). [14, 15].

### 2.5 Lipid Analysis

Lipid bodies were stained with Nile red and observed with the Nikon Optiphot 300 microscope (Nikon Instruments, USA) coupled with a Canon EOS Rebel T3 camera (Canon Industries, USA). For immobilization of the cells, 1 % agarose gel was prepared, poured into polystyrene rectangular plates (Corning), and allowed to

set. Actively growing culture (2 mL) was centrifuged in Eppendorf tubes, and the supernatant was discarded while the pellet was suspended in 1 mL deionized water. Nile red stain (10  $\mu$ L of 0.1 mg/mL Nile red powder in acetone) was added to 1 mL portion of the cell cultures, mixed by rapid inversion, and incubated in dark conditions for 20 min at room temperature [16]. The mixture (10  $\mu$ L) was dropped on the agarose gel, covered with cover slips, and viewed using both light and dark phases of the microscope. Total lipids were extracted from dried microalgal biomass (20 mg) using a modified Bligh and Dyer method. Biomass was pre-soaked in Milli-Q water, extracted with chloroform–methanol (1:2), phase-separated with chloroform and water, and centrifuged. The chloroform layer was collected, re-extracted, pooled, and vacuum-dried for gravimetric lipid determination. Method performance was evaluated using lipid spikes, with recoveries ranging from 85 – 98% and relative standard deviation (RSD) < 7%. The practical detection limit for gravimetric lipid measurement was approximately 0.5–1.0 mg [17].

## 2.6 C H N & S Analysis

The determination of the Carbon, Hydrogen, Nitrogen, and Sulphur content of the biomass employed the use of CHNS/O Analyzer (PerkinElmer 2400 series II) to combust the biomass. This gave the Carbon, Hydrogen, Nitrogen, and Sulphur components of the biomass. Freeze-dried samples between 2 – 10 mg were weighed using a microbalance (PerkinElmer AD 6000) into pre-weighed aluminum capsules (PerkinElmer) and sealed. These were combusted using helium as the carrier gas and cysteine as the standard [18].

## 2.7 In-situ Transesterification and FAMES Analysis

Biodiesel refers to fatty acid methyl esters (FAMES) produced by methyl esterification from triglyceride, a dominant component of algal lipid [19]. An acid-based reaction was carried out for both bound and free fatty acids. The freeze-dried microalgae (50 mg) were weighed and placed into a clear Agilent GC vial, and 20  $\mu$ L of C13:0 (10 mg/mL) internal standard was added. After shaking, 200  $\mu$ L of chloroform: methanol (2:1 v/v) and 300  $\mu$ L of 0.6 M sulfuric acid were added, and the vials were capped. The vials were heated in an oven set at 80 °C for 15 min with occasional vortexing. It was allowed to cool to room temperature, and 1000  $\mu$ L of high-grade hexane was added; then the top phase (100  $\mu$ L) was gently removed and diluted with 400  $\mu$ L of new hexane (1:5). This was injected into GC/MS for analysis [20]. The stock solution of the FAME mixture was prepared by diluting 10 mg in 1 mL of hexane, and this was run as a standard with the samples prepared.

## 2.8 GC–MS Analysis of Fatty Acid Methyl Esters (FAMES)

FAME analysis was performed using a gas chromatography–mass spectrometry (GC–MS) system

operated in scan mode. A 2  $\mu$ L sample was injected in splitless mode at an injector temperature of 230 °C. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min (linear velocity 5.40 cm/s). The oven temperature program was as follows: initial temperature of 100 °C, ramped to 200 °C at 25 °C/min and held for 1 min, further increased to 230 °C at 5 °C/min and held for 7 min, then ramped to 240 °C at 5 °C/min with a final hold of 2 min, giving a total run time of 23 min. The mass spectrometer was operated with a solvent delay of 2.60 min, an ion source temperature of 250 °C, a quadrupole temperature of 150 °C, an electron multiplier voltage of 2306 V, and a mass scan range of  $m/z$  50–650 at a scan rate of 2.34 scans/s. The MS outlet operated under high vacuum.

FAMES were identified by comparing their mass spectra with those in the NIST Mass Spectral Library and by matching retention times with authentic FAME standards where available. Compound identification was accepted at a spectral similarity match of  $\geq 85\%$ . Peak integration and relative abundance of individual fatty acids were determined from the total ion chromatograms.

## 3 Results and Discussion

### 3.1 Microalgae Isolates and their NCBI accession numbers

The microalgae isolates and their codes include: *Micractinium* sp. MOW 1 (NM 1), *Chlorella sorokiniana* MOW 2 (NM 2), *Chlorella* sp. MOW 3 (OJ 1), *Chlorella* sp. MOW 4 (OJ 2), *Scenedesmus* sp. MOW 5 (SH 1), *Coelastrrella* sp. MOW 6 (SH 2), *Stichococcus* sp. MOW 7 (SH 3), *Chlorella sorokiniana* MOW 8 (AB 1), *Graesiella emersonii* MOW 9 (AB 2), *Chlorella* sp. MOW 10 (IL 1), *Chlorophyta* sp. MOW 11 (IL 2), and *Chlorella* sp. MOW 12 (IL 3). NCBI accession numbers MT 735058 – 94.

### 3.2 Microalgae Culture in Photobioreactor



**Fig. 1.** Mini photobioreactors with filtered CO<sub>2</sub> inlet and O<sub>2</sub> outlet (Covered with filters) for effective gas exchange. The setup was placed in an illuminated incubator.

### 3.3 Protein, Carbohydrate and Lipid Content

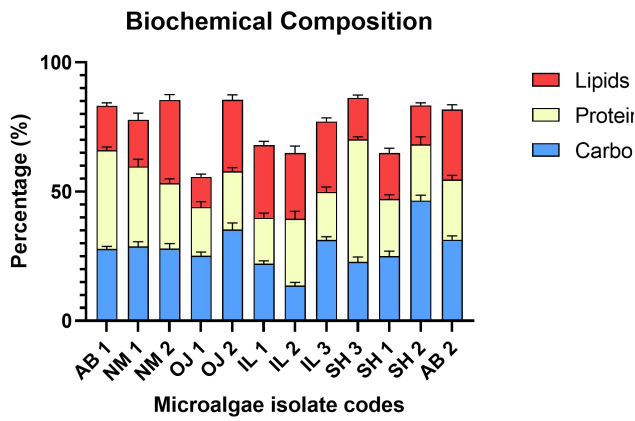


Figure 2: The percentage quantity of protein, carbohydrate, and lipid content of the microalgae isolates

### 3.4 C, H, N & S COMPONENTS

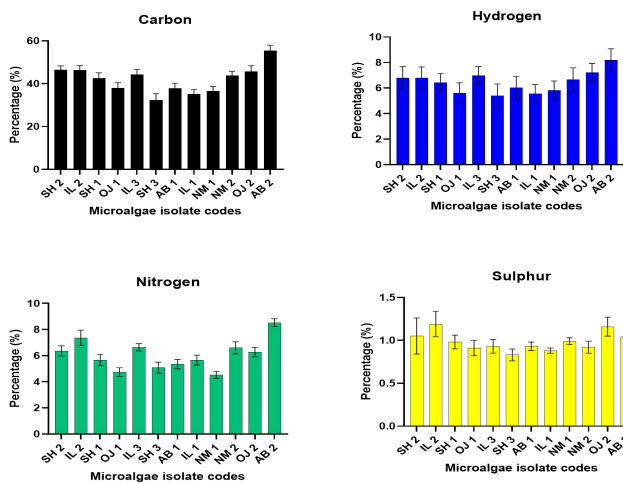


Fig. 3. The percentage carbon, hydrogen, nitrogen and sulphur components of the microalgae species.

### 3.5 OIL AND FAMES ANALYSIS

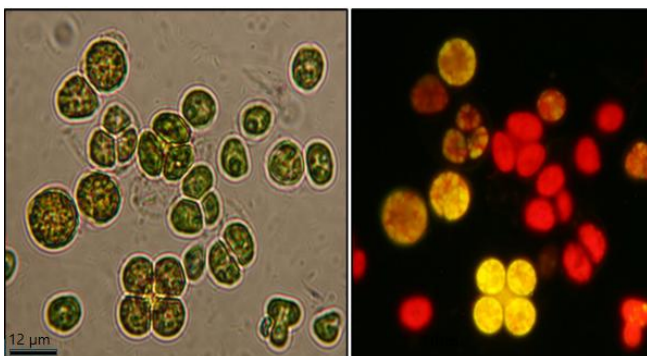


Fig. 4. The light and phase contrast microscope image of *Scenedesmus* sp. MOW 5

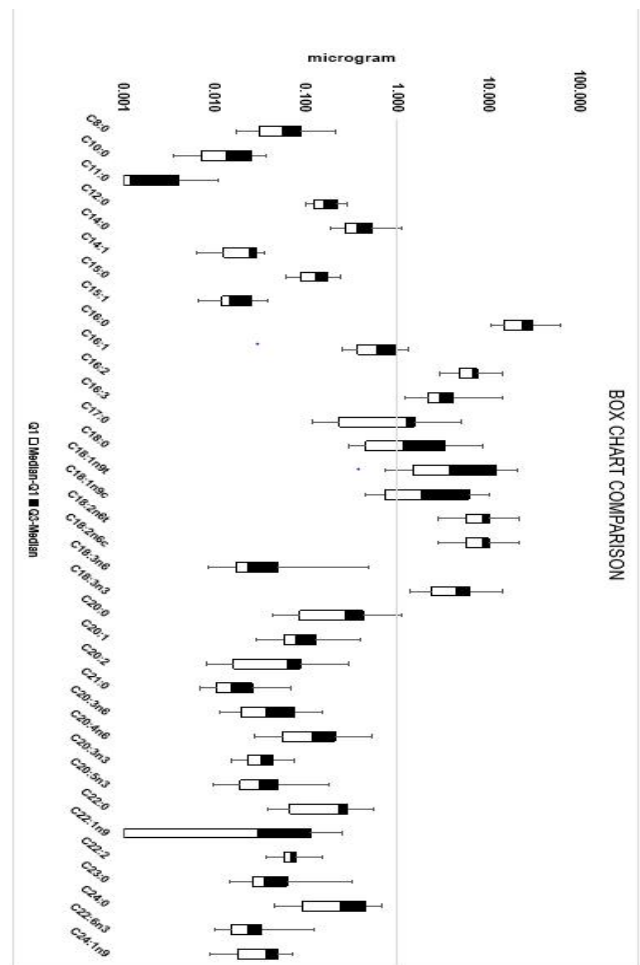


Fig. 5. A chart comparing the fatty acids (C8 – C24) identified with its average abundance in the microalgae species isolated

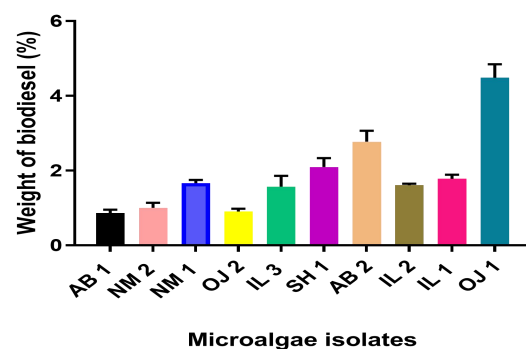


Fig. 6. The percentage of transesterified lipid in these microalgae species

## 4 Discussion

The findings of this study highlight the considerable potential of indigenous green microalgae as a sustainable feedstock for biodiesel production. The biochemical characterization of twelve isolates revealed significant variability in protein, carbohydrate, lipid, and elemental

composition, underscoring the metabolic diversity of these species and their suitability for multiple bio-based applications ranging from biofuels to feed supplements.

The protein content, ranging from 17.76% in *Chlorella* sp. MOW 10 to 47.43% in *Stichococcus* sp. MOW 7, indicates that several strains may serve dual purposes. While the primary focus was biodiesel production, their relatively high protein levels also position them as promising candidates for aquaculture feed or functional additives, thus supporting a biorefinery approach. However, as Ma and Hu [21] noted, the large-scale use of microalgae as protein sources for aquafeeds remains challenging due to high cultivation costs, although niche applications in enhancing feed performance remain attractive.

Carbohydrate content also varied widely (13.59 – 46.56%), and *Coelastrrella* sp. MOW 6 demonstrated notably high levels, comparable to values reported in earlier studies. Thepsuthamarat et al. [22] reported 46.71% carbohydrate, while Razoooki et al. [23] reported 50.7% carbohydrate in *Coelastrrella* species. Such carbohydrate-rich strains may be exploited not only for bioethanol production but also as substrates for microbial fermentation processes, expanding their bioenergy relevance beyond biodiesel. The dual-use potential of isolates with high carbohydrate and lipid fractions further strengthens the economic case for integrated valorization strategies.

Lipid content, the most critical factor for biodiesel, showed significant diversity across isolates, ranging from 11.74% to 32.17%. Among these, *Chlorella sorokiniana* MOW 2 stood out with the highest lipid yield, aligning with previous literature that identifies *Chlorella* species as among the most promising microalgae for oil accumulation. While these values are lower than the 47.5 – 55.1% lipid content reported in optimized commercial strains [24, 25], they remain within the range considered viable for biodiesel feedstock.

The CHNS analysis reinforced the role of microalgae as effective carbon sinks, with *Graesiella emersonii* MOW 9 recording the highest carbon content (55.55%). This not only highlights the carbon capture potential of these species but also aligns with global sustainability goals by coupling biofuel production with climate mitigation [26]. Elevated nitrogen levels in certain isolates also suggest potential for nitrogen recycling in wastewater-fed cultivation systems [27], further enhancing their environmental and economic appeal.

The presence of lipid bodies in the microalgae cells was visualized using the phase contrast microscope. These lipid bodies showed as yellow coloration, while the red coloration depicts the chloroplast cells. For some microalgae, formation of lipid bodies shows as a gradual change in the coloration from red – pink – orange – yellow while for others, it starts from a small part and enlarges until it fills the entire cell [28]. Notably, the lipid profiles revealed abundant quantities of palmitic

acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2), fatty acids widely recognized for producing biodiesel with favorable combustion, viscosity, and oxidative stability properties. These fatty acids were also reported in *Scenedesmus obliquus* by Sakthimurugan et al. [29] The predominance of saturated and monounsaturated fatty acids further enhances the quality of biodiesel derived from these indigenous isolates.

In terms of biodiesel yield, in-situ transesterification and GC-MS analysis confirmed the presence of diverse fatty acid methyl esters (FAMES), and *Chlorella* sp. MOW 3 (OJ 1) produced the highest yield (4.48 % of biomass). *Chlorella* sp. and *Coelastrrella* sp. [30] and *Chlorella* sp, and *Scenedesmus* sp [31] showed high biodiesel yield per percentage dry weight of biomass used (89 – 96 %). This could be attributed to strain development, nutrient limitation, heavy metal stressors, or environmental stress. While overall yields were modest compared to optimized benchmarks, the diversity of FAMES identified underscores the biochemical richness of these isolates. The detection of both medium-chain and long-chain fatty acids (C8–C24) suggests potential applications beyond fuel, including nutraceuticals and specialty chemicals, if refined biorefinery pathways are adopted [32].

Pearson correlation analysis revealed that lipid content exhibited a significant negative correlation with the carbon-to-nitrogen (C/N) ratio of the microalgal isolates ( $r = -0.616$ ,  $p = 0.033$ ), indicating that approximately 38% of lipid variability was explained by C/N balance ( $R^2 = 0.379$ ). In contrast, protein content showed a weak and statistically insignificant negative correlation with C/N ratio ( $r = -0.127$ ,  $p = 0.693$ ), while carbohydrate content displayed a moderate but non-significant positive correlation ( $r = 0.342$ ,  $p = 0.277$ ). The statistically significant lipid–C/N relationship confirms that: C/N balance is a major biochemical driver of biodiesel feedstock quality, and lipid accumulation is C/N-sensitive, even when protein and carbohydrate are not. This strengthens the biodiesel relevance of the CHNS dataset.

## 5 Conclusion

This study demonstrates that indigenous green microalgae isolated from local freshwater environments possess strong potential as sustainable feedstocks for biodiesel production. Comprehensive biochemical characterization revealed substantial variability in protein, carbohydrate, lipid, and elemental composition across the twelve isolates, highlighting their metabolic diversity and suitability for integrated biorefinery applications. Lipid content ranged from 11.74% to 32.17%, with *Chlorella sorokiniana* MOW 2 and *Chlorella* sp. MOW 3 is emerging as a promising candidate based on lipid yield and FAME profiles. The predominance of palmitic, oleic, and linoleic acids further supports the production of biodiesel with desirable combustion and stability properties.

The strong negative correlation between lipid content and the C/N ratio confirms the importance of nutrient balance in modulating lipid biosynthesis, suggesting that targeted manipulation of culture conditions can enhance biodiesel productivity. CHNS analysis additionally revealed high carbon fractions in several isolates, supporting their dual role in biomass generation and carbon sequestration.

Overall, the results underscore the feasibility of utilizing indigenous green microalgae for renewable biodiesel production while offering valuable co-products such as proteins and carbohydrates for feed and bioenergy applications.

## 6 Future Perspectives

Future work should prioritize optimizing cultivation conditions—particularly nutrient limitation strategies, CO<sub>2</sub> supplementation, and controlled stress induction—to further enhance lipid accumulation in the most promising strains. Scaling up production through cost-effective photobioreactor designs, wastewater-based cultivation, and two-stage bioprocessing approaches also warrants investigation. Advances in metabolic engineering and adaptive laboratory evolution may further improve lipid productivity and FAME quality. Integrating these strains into circular bioeconomy frameworks, including aquaculture feed systems and biorefinery pathways, could maximize economic viability and support broader sustainability goals.

Overall, this study underscores that indigenous green microalgae hold significant promises as renewable biodiesel feedstocks. Their metabolic diversity supports integrated biorefinery models where proteins, carbohydrates, and lipids are co-valORIZED, enhancing both sustainability and economic feasibility. By leveraging advances in cultivation technology and bioprocess optimization, these strains can make a meaningful contribution to the global transition toward low-carbon, renewable energy sources, aligning with the Sustainable Development Goals on climate action, energy security, and environmental health.

## 7 Acknowledgement

The authors acknowledge the support from the Tertiary Education Trust Fund (TETFund) Institutional-Based Research grant, Nigeria.

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